REMARKS

Claims 1, 3, 4, 7-10, 12, 13 and 16-20 are presently pending in this application. In the instant Amendment, claims 1 and 18 have been amended by adding the term "*wherein the signal-to-background ratio achieved from the growth marker reporter activity multiplies with the signal-to-background ratio achieved from the enzyme reporter activity*" as disclosed on page 10, second paragraph to page 11, second paragraph of the specification. Furthermore, claim 18 has been amended to clarify that the promoters of the two reporter genes are the same, as supported by the Examples where same promoters are used (see e.g. page 16, line 2 of the specification).

Claim 10 has been amended to refer back to measuring step d], a correction required by the Examiner.

Rejections under 35 U.S.C. § 103

Claims 1, 3, 4, 7-10, 12, 13, 16, 17, 19 and 20 are rejected under 35 U.S.C. 103(a) as over Keating et al., (Oncogene 20:4281-4290 (2001))("Keating") in view of Brown et al., (Yeast 16:11-22 (2000))("Brown") and in further view of Yang et al., (J. Biol. Chem. 273(14):8212-8216 (1998))("Yang") and Tanaka *et al.,* (Annals of Thoracic Surgery, 72:1173-1178 (2001))("Tanaka").

In the Examiner's view, "*Applicant claims a method of identifying an agent that modulates the activity of a target molecule wherein the agent contacts a cell and modulates the target molecule, and wherein said cell also comprises two reporter genes. After contact by agent, cell propagation and reporter activity are measured. One of the reporter genes produces an enzyme, and the substrate of the enzyme and a substance capable of permeabilizing the membrane are added after a delay, specifically at least two cell cycles. The target molecule affects the reporter gene, and is further limited to a heterologous molecule and can be a nucleic acid or polypeptide. The target molecule affects cellular propagation indirectly or through an intermediary molecule. The target molecule can also affect the reporter gene and cellular propagation directly. The reporter gene produces an enzyme whose activity*

is detectable on the basis of conversion of a substrate. The cell is a yeast cell, specifically S. cerevisiae.

Applicants have amended claims 1 and 18 to more clearly describe the invention. The phrase "*wherein the signal-to-background ratio achieved from the growth marker reporter activity multiplies with the signal-to-background ratio achieved from the enzyme reporter activity*" has been added to both claims. Support for this amendment can be found in the specification on page 10, second paragraph through page 11, second paragraph. Amended claim 1 now reads as follows: A method utilizing a double reporter assay for improving signal-to-background ratio to identify an agent which modulates activity of a target molecule, wherein said target molecule affects cellular propagation, said method comprising the steps of: a] contacting a cell with a candidate compound, wherein said cell comprises said target molecule, and wherein said cell further comprises a growth marker reporter gene and a gene coding for enzyme whose activity is detectable on the basis of conversion of a substrate; b] adding said substrate and a substance capable of permeabilizing the membrane of said cell with a delay after said contacting step a]; c] measuring cell propagation; and d] measuring activity of said reporter genes, wherein said target molecule affects the activity of said reporter genes, and wherein the signal-to-background ratio from the growth marker reporter activity multiplies with the signal-to-background ratio from the enzyme reporter activity.

The Examiner's reliance on Keating is misplaced. Keating has been cited by the Examiner as allegedly teaching one feature of the present invention wherein the substrate of a reporter enzyme is added with delay (cf. page 6, 2nd line of the Office Action). However, the luciferase assay as described in the 5th paragraph of the "Materials and methods" constitutes a totally different approach from the one described in the present invention. More specifically Keating *et al.*, does not combine growth of the cells with the production of a product produced by the activity of an enzyme (luciferase activity). Moreover, Keating use mutant ATM promoters for investigating Sp1 involvement as disclosed on page 4284, second paragraph. This results in a dramatic reduction of luciferase activity. Thus, Keating uses a promoter sequence which abolishes expression whereas in the present invention, expression of the enzyme is necessary for investigating the influence of a test compound. As a result, Keating discloses

measurement of luciferase activity in a context very different from that of the present invention. The skilled person would therefore not have an incentive to consider Keating whose disclosure teaches away from the present invention.

The present method uses a combination of measurements, one based on a logarithmic event, which is growth, with another based on a separate linear event such as enzyme activity. The combination of logarithmic and linear events leads to enhanced levels of enzyme activity, based on the fact that more cells produce more enzyme molecules. This aspect has to be considered independent of the fact at what time point luciferase activity is measured. By combining growth with enzyme activity, the signal-to-background ratio is multiplied when the resulting measurements of enzyme activity and cell propagation as claimed are taken.

Only the present inventors have discovered that if combining an assay based on growth with an assay based on conversion of a substrate, the signal-to background ratio can be markedly improved. This result depends on the fact that the enzyme activity is measured after finishing growth by using a cell permeabilizing agent. The necessity for such a separation of growth and measurement of enzyme activity is not obvious from Keating. Thus, the skilled person, if reading the document of Keating would not obtain an incentive to determine enzyme activity using cell extracts after incubation with EGF for enhancing the signal-to-background ratio. Moreover, combining growth and enzyme activity renders the enzyme activity dependent on growth, so that enzyme activity is a measure of growth resulting in a highly sensitive assay.

Brown et al., (Yeast 16:11-22 (2000))("Brown")

The Examiner acknowledges that Keating does not teach the use of two different reporter genes nor of the use of a membrane permeabilizing agent to facilitate enzyme substrate conversion. The Examiner contends that Brown teaches the use of a dual reporter assay for evaluating chimeric yeast/mammalian G alpha proteins. As already indicated in our previous response to an Office Action, Brown has not realized the problem that if adding the substrate together with a compound for stimulation of the receptor, the substrate can (1) reach the interior

of the cell through the plasma membrane only with difficulty and (2) the substrate which reaches the cell interior may be inhibited. Thus, even if there exist efforts in the prior art to increase signal-to-background ratios by combining several reporter assays, as alleged by the Examiner, there is no teaching in the prior art of how to combine two or more single reporter assays in order that the signal-to-background ratio achieved by the single reporter assays multiply as is achieved by the present invention. As discussed on page 10, last paragraph of the applicant's specification, where it is shown that the growth reporter assay which has a signal-to-background ratio of 30-50:1 and the enzyme reporter assay with an increase of signal-to-background ratio of 2-3 times multiply to give a ratio of 100-150:1. It could be shown by the present inventors that simply combining such assays without considering of how to apply the substrate for measuring enzyme activity may not result in the expected increase of the signal-to-background ratio. Brown has not realized that such a high increase of signal-to-background ratio can be obtained by separating addition of test compound and substrate. On the contrary, the skilled person would not have an incentive to change the assay of Brown et al. without an indication that efficiency could be increased by applying the substrate after the contacting step with the test compound. This is due to the fact that there is no disclosure in Brown that the concomitant use of substrate and incubation of cells have impaired efficiency. Brown disclose that CPRG is cell-permeant (see page 14, first paragraph), which implies that such assay would be as efficient as if the substrate is applied after growth with a permeabilizing agent.

The person skilled in the art would not turn to Keating in order to overcome the above problem as Keating was not concerned with the double reporter assay using a growth-based assay and an enzyme-based assay and because Keating's approach involved a mutant, non-active promoter.

Tanaka *et al.*, (Annals of Thoracic Surgery, 72:1173-1178 (2001))("Tanaka")

The Examiner holds the view that the skilled person would be motivated to use Tanaka (newly introduced by the Examiner) as this document discloses the use of digitonin for increasing cellular permeability and intracellular uptake.

As stated above, a combination of Brown and Keating is not suitable to suggest the present invention. Brown discloses the addition of substrate during the entire period of stimulation of the receptor with ligand. It is stated on page 11, lines 7 and 8 of the present specification that CPRG can reach the cell interior only with difficulty. Applying Tanaka on Brown would mean that the skilled person would apply digitonin for increasing cell permeability in order to increase uptake of CPRG by the cells during the entire period of stimulation. However, such an action is not envisaged by the present inventors who, by separating addition of test compound and substrate, also addressed the problem that CPRG is inhibited during ligand-induced growth. Keating disclose the addition of substrate to a cell extract in a single reporter assay using a promoter construct which inactivates luciferase activity. Thus, a combination of the Keating, Brown and Tanaka is not suitable to suggest the present invention.

Yang et al., (J. Biol. Chem. 273(14):8212-8216 (1998))("Yang")

The approach used by Yang is totally different from the approach of the present invention. In the present invention, the problem to be solved is to increase the signal-to-background ratio by combining a logarithmic or growth-based reporter assay with a linear or enzyme-based reporter assay which renders growth of cells and enzymatic production of a product by an enzyme dependent on the activity of a candidate compound. In Yang, fluorescent proteins are used for monitoring gene expression and protein localization. Thereby, a variant of GFP termed EBFP is used to improve the efficiency of chromophore formation. Yang could show that the variant results in an enhanced fluorescence. Yang also provide a dual reporter assay using, in addition to EBFP, the fluorophore EGFP, however, not, as alleged by the Examiner, to improve the signal-to-background ratio but to detect the activity of different promoter elements or to detect localization of two different proteins (cf. page 8216, 2nd paragraph). Increasing the signal-to-background ratio as alleged by the Examiner would mean, if applied to Yang that the colours as produced by the fluorescent proteins would overlay and multiply. This is, however, not the case. A dual reporter assay using different fluorophores can only be suitable to detect different proteins or to detect different localizations. Thus, reading the document of Yang the skilled person would not obtain an incentive to combine a growth-

based reporter assay with an enzyme-based reporter assay which multiply in their efficiency as the object of Yang is totally different from the object of the present invention, thus not contributing to a solution of the problem solved by the present invention. Moreover, as the extent of gene expression is monitored by fluorescence, there is no disclosure with respect to an assay format for measuring enzyme activity. Thus, the combination of Keating, Brown, Tanaka and Yang are not suitable to suggest the present invention as presently amended.

Claim 18 is rejected under 35 U.S.C. 103(a) as over Crossin et al., (PNAS 94:2687-2692 (1997)) ("Crossin") in view of Keating, in view of US 6,063,578 and in further view of US 20050118690 to Roberts et al. ("Roberts")and Tanaka.

Claim 18 has been amended to further clarify the invention. Support for the amendment can be found throughout the specification, including the Examples where the same promoters are used and in particular on page 16, line 2 of the specification.

Amended claim 18 is directed to a method of identifying an agent which modulates the activity of at least one target molecule, wherein said at least one target molecule affects cellular propagation, said method comprising the steps of: (a) contacting a first cell with a candidate compound, wherein said first cell comprises a first target molecule, and wherein said cell further comprises a growth marker reporter gene and a gene coding for an enzyme whose activity is detectable on the basis of conversion of a substrate under the control of the same promoter as used for control of the growth marker reporter gene; (b) contacting a second cell with a candidate compound, wherein said second cell comprises a second target molecule, and wherein said cell further comprises a growth marker reporter gene and a gene coding for enzyme whose activity is detectable on the basis of conversion of a substrate under the control of the same promoter as used for control of the growth marker reporter gene; (c) adding said substrate and a substance capable of permeabilizing the membrane of said cell with a delay after said contacting steps (a) and (b); (d) measuring cell propagation of said first cell; (e) measuring cell propagation of said

second cell; (f) measuring activity of said reporter genes in said first cell; and (h) measuring activity of said reporter genes in said second cell, wherein said at least one target molecule affects the activity of said reporter genes and wherein the signal-to-background ratio from the growth marker reporter activity multiplies with the signal-to-background ratio from the enzyme reporter activity.

Crossin et al., (PNAS 94:2687-2692 (1997)) ("Crossin")

The Examiner alleges that Crossin teach the present invention in that Crossin teaches that an agonist, N-CAM, modulates a target molecule, GRE, which induces a luciferase reporter. Moreover, according to the Examiner, Crossin allegedly teach a second cell with a second target molecule, CMV, and a second reporter β-galactosidase.

However, the interpretation of Crossin by the Examiner is not quite correct. As is obvious from page 2688, right hand column, 3rd and 4th paragraphs, and Figure 4, GRE-luc and CMV-β-gal vectors are not electroporated into a first and second cell but are electroporated into one cell, whereby transfection with CMV-β-gal serves normalization of luciferase activity. Thus, Crossin do not teach the subject-matter of claim 18 which is directed to a multiplex assay in which a first and a second cell with different target molecules are used in order to simultaneously investigate the influence of an agent on different target molecules. In Crossin the same agent, N-CAM, is used and its influence on the same target molecule which directly or indirectly influences the transcription of reporter genes, namely the luciferase gene and the β-galactosidase gene, under the control of different promoters, namely GRE and CMV, is investigated. Thus, Crossin is not useful in suggesting the present invention.

The fact that Crossin prepares cell lysates prior to measuring enzyme activity cannot heal the above mentioned deficiency of Crossin who does not address the issue of a multiplex assay and who does also not address the necessity of same promoters for growth and enzyme reporter measurement. As discussed above, Claim 18 is directed to an assay wherein a first cell and a second cell are contacted with a candidate compound whereby both cells harbour dual reporter genes for growth-based and for enzyme-based assays and use, for detection of the enzyme

activity, permeabilization of the membrane by a substance and concomitant addition of the substrate after the contacting step of the candidate compound with the cell. Moreover, the promoter elements for regulating expression of the reporter genes are the same.

Keating and Tanaka

Crossin does not teach the use of a multiplex assay using different cells with different target molecules and do not teach cells wherein the promoters of the growth marker and enzyme genes are identical. Consequently, the teachings of Keating and Tanaka which are directed to enzyme measurement and permeabilisation of cells are not suitable to suggest claim 18 (see also the discussion above).

US 6,063,578

Regarding US patent 6,063,578, the Examiner holds the view that this document is relevant for the present invention as it teaches a dual reporter assay using different reporters. US 6,063,578 is different from the present invention in that the system disclosed therein comprises dual reporters for use in the independent evaluation of transcription and replication. In column 6, the reporter system for evaluation of transcription of a reporter gene is disclosed. Thereby, a regulatory sequence is linked to a first reporter gene. The object is that the transcription of a linked gene should be changed and this change is measured. The second reporter plasmid is designed to permit an evaluation of plasmid replication (cf. column 7). The object is not to modulate the transcription of the gene but the replication of the plasmid whereby the level of expression of the reporter protein is indicative of the level of plasmid replication. Examples of regulatory sequences are in column 7, first and second paragraphs. The sequences mentioned are viral sequences.

The disclosure of US 6,063,578 stands in contrast to the presently amended claim 18, wherein both reporter genes are under the control of same promotor sequences. Thus, the growth marker reporter gene and the enzyme gene are expressed to an equal extent. In US 6,063,578, transcription of a reporter gene and plasmid replication are evaluated requiring the use of different regulatory sequences which does not necessarily result in a uniform expression of both

reporter genes. The Examiner is directed to column 2, lines 1 to 4 and column 7, lines 42 to 48, where it is stated that the effect of the transcriptional regulator on the second reporter gene should be much less than on the transcriptional reporter gene. In other words, transcription and replication should be independent. This independence of transcription and replication is not in line with the present invention where the promoters of both genes are the same so that the influence of the transcriptional regulator of the target signalling pathway on expression of the growth reporter and enzyme gene is the same and allows expression of both genes to an equal extent. Thus, both systems in one cell supplement each other which results, in addition to the enzyme activity as achieved by the influence of the promoter per se, in a modulated enzyme activity dependent on the growth of the cell, thus uniformly reflecting the degree of growth.

This constitutes a difference between both inventions whereby US 6,063,578 is not suitable to suggest the double reporter assay which has as an object to investigate the activity of an agent which modulates propagation and enzyme activity, whereby growth and enzyme activity are uniformly regulated so that the extent of enzyme activity finally is also an expression of growth.

US 20050118690 to Roberts et al. ("Roberts")

Roberts teaches a dual reporter assay for isolating transformants expressing improved variant regulator proteins. Despite the alleged disclosure of a dual reporter assay with one reporter gene providing a growth advantage and the other reporter gene providing a colorimetric marker, this document is directed to the creation of a plurality of improved variant regulator proteins. This object is totally different from the object of the present invention which is the identification of an agent which modulates a target gene affecting cellular proliferation.

Moreover, despite the alleged disclosure of a dual reporter assay, the authors of US 2005/0118690 do not disclose that for an efficient signal-to-background ratio, the substrate together with the substance for permeabilizating the membrane of the cell should be applied after the contacting step with the candidate compound. The advantage of such a procedure is

described in the present invention disclosure and is not obvious from US 2005/0118690. Without a disclosure in Roberts of the fact that the substrate should be added together with a substance for permeabilizing the membrane of the cell after the contacting step of the candidate compound, the skilled person is not provided with the information of how to best carry out the step of measurement of enzyme activity. The feature of the improved signal-to-background ration has been included into claim 18 thus distinguishing claim 18 from Roberts.

Furthermore, Roberts is silent with respect to a multiplex assay and is also silent with respect to the use of same promoter sequences for regulating reporter genes, the advantages thereof being described above.

Fees

No fees are believed to be necessitated by the instant response. However, should this be in error, authorization is hereby given to charge Deposit Account no. 18-1982 for any underpayment, or to credit any overpayments.

CONCLUSION

Applicants respectfully request entry of the foregoing amendments and remarks in the file history of the instant Application. The Claims as amended are believed to be in condition for allowance, and reconsideration and withdrawal of all of the outstanding rejections is therefore believed in order. Early and favorable action on the claims is earnestly solicited.

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